

# An iTRAQ-Based Quantitative Proteomic Analysis of Plasma Proteins in Preterm Newborns With Retinopathy of Prematurity

Magdalena Zasada,<sup>1</sup> Maciej Suski,<sup>2</sup> Renata Bokinić,<sup>3</sup> Monika Szwarc-Duma,<sup>3</sup> Maria Katarzyna Borszewska-Kornacka,<sup>3</sup> Józef Madej,<sup>2</sup> Beata Bujak-Giżycka,<sup>2</sup> Anna Madetko-Talowska,<sup>4</sup> Cecilie Revhaug,<sup>5</sup> Lars O. Baumbusch,<sup>5</sup> Ola D. Saugstad,<sup>5</sup> Jacek Józef Pietrzyk,<sup>1</sup> and Przemko Kwinta<sup>1</sup>

<sup>1</sup>Department of Pediatrics, Jagiellonian University Medical College, Krakow, Poland

<sup>2</sup>Chair of Pharmacology, Jagiellonian University Medical College, Krakow, Poland

<sup>3</sup>Neonatal and Intensive Care Department, Medical University of Warsaw, Warsaw, Poland

<sup>4</sup>Department of Medical Genetics, Jagiellonian University Medical College, Krakow, Poland

<sup>5</sup>Department of Pediatric Research, Oslo University Hospital, University of Oslo, Oslo, Norway

Correspondence: Przemko Kwinta, Department of Pediatrics, Jagiellonian University Medical College, Wielicka 265, Krakow 30-663, Poland; kwintap@mp.pl.

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**PURPOSE.** Retinopathy of prematurity (ROP) is a vision-threatening complication of a premature birth, in which the etiology still remains unclear. Importantly, the molecular processes that govern these effects can be investigated in a perturbed plasma proteome composition. Thus, plasma proteomics may add new insights into a better understanding of the pathogenesis of this disease.

**METHODS.** The cord and peripheral blood of neonates ( $\leq 30$  weeks gestational age) was drawn at birth and at the 36th postmenstrual week (PMA), respectively. Blood samples were retrospectively subdivided into ROP(+) and ROP(−) groups, according to the development of ROP.

**RESULTS.** The quantitative analysis of plasma proteome at both time points revealed 30 protein abundance changes between ROP(+) and ROP(−) groups. After standardization to gestational age, children who developed ROP were characterized by an increased C3 complement component and fibrinogen level at both analyzed time points.

**CONCLUSIONS.** Higher levels of the complement C3 component and fibrinogen, present in the cord blood and persistent to 36 PMA, may indicate a chronic low-grade systemic inflammation and hypercoagulable state that may play a role in the development of ROP.

**Keywords:** retinopathy of prematurity, proteome, complement, fibrinogen

Retinopathy of prematurity (ROP) is a biphasic ocular disease occurring primarily in infants delivered before approximately 28 weeks gestational age with immature retina.<sup>1</sup> It includes a delayed physiologic retinal blood vessels development, followed by anarchic vessel proliferation and a potential for succeeding retinal detachment.<sup>2</sup> An initial vessel loss may evoke ischemic events with detrimental effects on neuronal homeostasis and functions.<sup>3</sup> Although early detection and treatment often result in a full recovery, severe cases may lead to long-lasting problems of reduced visual acuity, high myopia, strabismus, nystagmus, glaucoma, late retinal detachments, or even blindness.<sup>4–8</sup> Until now, the pathogenesis of ROP has not been completely understood. The various ROP risk factors include low gestational age and low birthweight, higher or fluctuating oxygen levels during oxygen therapy, hyperglycemia, insulin use, inadequate postnatal nutrition, improper insulin-like growth factor 1 concentration, poor postnatal growth, pre- and postnatal infection, and genetic factors.<sup>9</sup> However, we still know too little about why some infants develop this complication of prematurity while others, born at the same age and with similar clinical characteristics, do not. Studies on protein abundance profiling may not only broaden our knowledge about potential achievable biomarkers and help

enhance our understanding of the molecular process driving ROP but also explain the susceptibility of some preterm newborns to ROP. Up to now, limited research has identified several proteins (i.e., these from the insulin-like growth factor pathway, mitochondrial superoxide dismutase, and chordin-like protein 1), in which altered levels in plasma were related to the increased risk of ROP.<sup>10,11</sup> A study dealing with vitreous samples of children with ROP has also revealed some proteins potentially involved in the pathomechanism of ROP.<sup>12</sup> However, the limited material availability makes these studies more relevant in basic science but not in routine clinical practice.

In our previous publications, we described the comparison between abundances of all plasma proteins and differences at the transcriptional level (mRNA) from prematurely born children with different gestational ages, both from cord blood as well as at the 36th postmenstrual week (36 PMA).<sup>13–15</sup> We presented that protein abundance differences are highly dependent on gestation age.

The goal of this study was to identify potential ROP plasma biomarkers and provide a more molecular-based understanding of ROP by comparing a proteome profile at the two time points (at birth and at 36 PMA) in groups of infants with and without ROP. However, as our previous studies revealed, the level of



prematurity has a profound impact on the plasma protein quantitative changes. Therefore, we performed a standardization of the obtained results to the gestational age. Our secondary aim was to corroborate the protein level by a complementary gene expression study.

## METHODS

This study is an analysis of data collected as part of a multisite study to investigate the transcriptome and proteome in preterm infants.<sup>13–15</sup> The study was approved by the Jagiellonian University Bioethical Committee and adheres to the tenets of the Declaration of Helsinki.

### Enrolled Patients

All preterm newborns with a gestational age of  $\leq 30$  weeks were consecutively enrolled into the study between September 1, 2013, and November 30, 2015, at the Warsaw Medical University Neonatal Intensive Care Unit (NICU), Warsaw, Poland. Informed consent was obtained antenatally from the parents with an explanation of the nature and possible consequences of the study.

### Monitoring During Hospitalization

All the subjects enrolled in the study underwent careful clinical monitoring for symptoms of ROP, as the standard of care. The screening of infants for ROP followed standard guidelines.<sup>16</sup> The examination and the qualification for laser therapy were provided by an experienced specialist in ophthalmology and retina diseases. Laser photocoagulation was performed on children who developed any stage ROP with plus disease in zone I, stage 3 ROP without plus disease in zone I, or stage 2 or 3 ROP with plus disease in zone II. In the case of ROP progression despite laser treatment, the patient might have been qualified to receive anti-vascular endothelial growth factor therapy.

### Data Collection

Data concerning perinatal history, hospitalization course, and the patient's outcome, with special regard to the course and treatment of ROP, were simultaneously collected in an Excel database.

### Division Into Groups

All patients with eye changes typical for any stage of ROP<sup>17</sup> were included into the ROP(+) group. Patients without any eye abnormalities associated with ROP formed the ROP(–) group.

### Blood Sampling

Blood samples for transcriptome and proteome analyses were collected at two time points: during delivery (umbilical cord blood) and at 36 PMA peripheral venous blood. The blood samples were further managed as previously described in detail elsewhere.<sup>13–15</sup>

### Proteome Analysis

ProteoMiner beads (combinatorial peptide ligand library beads; Bio-Rad, Hercules, CA, USA) were used for the enrichment procedure, optimized with reference to previously published protocols.<sup>18,19</sup> The quantitative analysis was performed by isobaric tag for a relative and absolute quantitation (iTRAQ) method (Sciex, Framingham, MA, USA). Samples were en-

riched, trypsin-digested, randomly assigned to iTRAQ reagents, labeled, and, finally, combined to corresponding 8plex assemblies. For data normalization, each 8plex assembly contained an internal common reference generated by combining equal amounts of protein from all of the samples included in the measurements. Next, labeled peptides were fractionated by strong cation exchange chromatography, after which flow-through fraction and 11 consecutive injections of the elution buffer (5–500 mM ammonium acetate) were collected. Thus, the labeled peptides from each 8plex assembly were distributed across 12 strong cation exchange fractions. Each fraction was then separated by reversed-phase liquid chromatography and applied on-line to a Velos Pro (Thermo Scientific, Waltham, MA, USA) mass spectrometer through a nanoelectrospray ion source. Spectra were collected in full scan mode (400–1500 Da), followed by five pairs of collisional-induced dissociation (CID) and higher energy collisional dissociation (HCD) tandem mass spectrometry (MS/MS) scans of the five most intense precursor ions from the survey full scan and, subsequently, merged to hybrid HCD-CID spectra by EasierMGF software.<sup>20</sup> These were analyzed by the X!Tandem (The GPM Organization)<sup>21</sup> and Comet<sup>22</sup> search engines, statistically validated with PeptideProphet, and integrated with iProphet<sup>23</sup> under the Trans-Proteomic Pipeline (TPP) suite of software (Institute for Systems Biology, Seattle, WA, USA).<sup>24</sup> The peptide false discovery rate (FDR) was estimated by Mayu<sup>25</sup> (TPP), and peptide identifications with an FDR below 1% were considered to be correct matches. Imputation of the missing values in peptide abundances was performed in a MaxQuant environment<sup>26</sup> on the log<sub>2</sub>-transformed normalized iTRAQ, which reports intensities with a criterion of at least 75% of the values present for a peptide in the dataset by drawing the values from the normal distribution, with parameters optimized to mimic a typical low-abundance measurement. DanteR software<sup>27</sup> was used for protein quantitation and the statistical analysis of iTRAQ-labeled peptides. ANOVA was performed at the peptide level by using a linear model with the Benjamini and Hochberg FDR correction used to adjust *P* values. Protein fold change was reported as a median value of corresponding unique peptides.

### Justification of Sample Size

A formal sample size calculation was not performed so as to allow the realization of a hypothesis-generating study. The approximate sample size was based on the calculations for the main study.<sup>13</sup> Briefly, the basic goal of the main project was to compare protein abundance levels between groups with a different degree of maturity. The power analysis (<http://www.dssresearch.com/toolkit/default.asp>; provided in the public domain by DSS Research, Fort Worth, TX, USA) indicated that with *n* = 19 patients in each preterm group, the estimated power of the study to validate the measured fold change at the level of 1.15 was 0.98 (*P* = 0.05). Therefore, *n* = 19 patients were enrolled to each preterm subgroup. Using the calculation mentioned above, we can state that the power of this study is at least that in the publication mentioned above; we can detect at least a 1.15-fold change in protein abundance between ROP(–) and ROP(+) patients.

### Microarray Analysis

To study the whole-genome expression with a comprehensive coverage of proteins by appropriate gene transcripts, SurePrint G3 Human Gene Expression v3, 8 × 60 K microarrays (Agilent, Santa Clara, CA, USA) were used. A detailed methodology of handling the samples was described previously.<sup>15</sup> A microarray gene expression experiment was performed according to the

**TABLE 1.** Comparison of Selected Demographic Variables and Hospitalization Data of the Patients in the Studied Groups

Variables	ROP(−) Group, <i>n</i> = 29	ROP(+) Group§, <i>n</i> = 28	<i>P</i> Value
Gestational age in weeks, mean (SD)	28 (1)	26 (2)	<0.001*
Birthweight in g, mean (SD)	1097 (245)	904 (220)	0.003*
Female sex†	18 (62.1)	20 (71.4)	0.576‡
Antenatal steroids†	23 (79.3)	21 (75)	0.760‡
Clinical signs of chorioamnionitis†	7 (25)	14 (50)	0.097‡
Histologically confirmed chorioamnionitis†	7 (26.9)	14 (53.8)	0.089‡
Sepsis†	1 (3.5)	2 (7.1)	0.612‡
Intraventricular hemorrhage grade 3/4†	0 (0)	2 (7.1)	0.237‡
Oxygen therapy in days, mean (IQR)	11 (2–38)	51 (32–71)	<0.001§
On oxygen during 28th day of life†	12 (41.4)	24 (85.7)	0.001‡
On oxygen at 36 weeks postmenstrual age (PMA)†	1 (3.4)	6 (21.4)	0.052‡

\* Student's *t*-test.† Results are presented as *n* (%).

‡ Fisher's exact test.

§ Mann-Whitney *U* test.

manufacturer's protocol (Two-Color Microarray-Based Gene Expression Analysis, Low Input Quick Amp Labeling v6.9.1). After the experiment, the arrays were scanned by the SureScan Microarray Scanner (Agilent) and data were extracted using the Feature Extraction Software (Agilent). The expression of selected genes was confirmed by RT-PCR analysis as described in detail elsewhere.<sup>15</sup>

### Statistical Analysis

Descriptive statistics were calculated and compared across the groups using Student's *t*-tests or Fisher exact tests for variables such as gestational age and birthweight, sex, antenatal steroids, and selected neonatal complications, including chorioamnionitis and oxygen therapy at the 28th day of life and 36 weeks PMA. A Mann-Whitney *U* test was used to compare the length of oxygen therapy between the groups. The groups determined by the comparison of interest were not comparable with respect to gestational age. Therefore, we wanted to examine whether differences in protein concentrations between the groups were due to gestational age. A linear model was fitted to the protein concentrations for each protein, and Student's *t*-tests and *F*-statistics were computed for each contrast, group indicator and gestational age. For each protein that was found to have a different concentration between the groups (i.e., that had a FDR-adjusted *P* value <0.05 in the first part of the analysis), we tested whether this expression was explained by the group indicator and/or by gestational age, using a logistic regression analysis. For statistical analysis, an SPSS software package (IBM SPSS Statistics for iOS, v24.0; IBM Corp., Armonk, NY, USA) was used.

### RESULTS

Fifty-seven preterm newborns were included in the study, and, with two time points of blood drawing, this resulted in the collection of a total of 114 samples for proteomic and transcriptomic analysis. During their hospitalization in the NICU, 28 infants who had developed ROP formed the ROP(+) group and 29 ROP-negative infants formed the second group (Fig.). The overall characteristics of the cohort and the differences in selected variables across the analyzed groups are shown in Table 1. Children who, during hospitalization, developed ROP were characterized by a lower gestational age and birthweight, and, moreover, they required more prolonged oxygen therapy.

In the first stage of the analysis, the concentrations of proteins in serum obtained from the cord blood of preterm born children were analyzed. The abundance level of 33 of the proteins for the group who, during hospitalization, developed ROP were significantly different when compared with preterm infants who did not have this complication (Table 2). The quantitative comparisons between samples collected at 36 weeks PMA from ROP(+) and ROP(−) groups revealed a significantly different abundance level of 30 proteins (Table 3). After adjustment for gestational age, complement C3 and fibrinogen alpha chain revealed a significant association with ROP at both time points. Moreover, at 36 PMA, the fibrinogen gamma chain revealed a significant, and the fibrinogen beta chain a borderline, association with ROP (Tables 2 and 3). Among the 28 children with ROP, 5 presented ROP at the stage which required laser photocoagulation. This subgroup generally consisted of the most immature infants: gestational age, 24.6 (SD 1.94) weeks; birthweight, 750 (SD 176) grams; female sex, 3 (60%); prenatal steroids, 3 (60%); sepsis, 1 (20%); intraventricular hemorrhage grade 3/4, 1 (20%); on oxygen at 28th day of life, 5 (100%); on oxygen at 36 weeks PMA, 2 (40%); clinical chorioamnionitis, 3 (60%); histologic chorioamnionitis, 3 (60%). No one required antivasculature endothelial growth factor therapy. In order to broaden the spectrum of observed changes to different biological level of regulation, the obtained results were compared in detail with the results of the gene expression microarray study. At both analyzed time points, significant differences for complement C3 component were observed (Table 4).

### DISCUSSION

Our study presents the results of plasma proteome analysis in infants who, after birth at ≤30 gestational weeks, subsequently developed or did not develop ROP. Our findings support previous literature by showing that lower gestational age and birth weight correlate with a higher risk of developing ROP during hospitalization. Moreover, ROP(+) and ROP(−) groups differed in the length of oxygen therapy as well as the proportion of children who developed bronchopulmonary dysplasia, which is also an oxygen-use-connected disease. Therefore, we consider oxygen therapy as the most significant predictor of ROP. Moreover, we identified two proteins whose increased level in cord blood plasma differentiate children with and without the risk of subsequently developing ROP. Additionally, these differences were also present in the examination of blood plasma obtained from the same infants

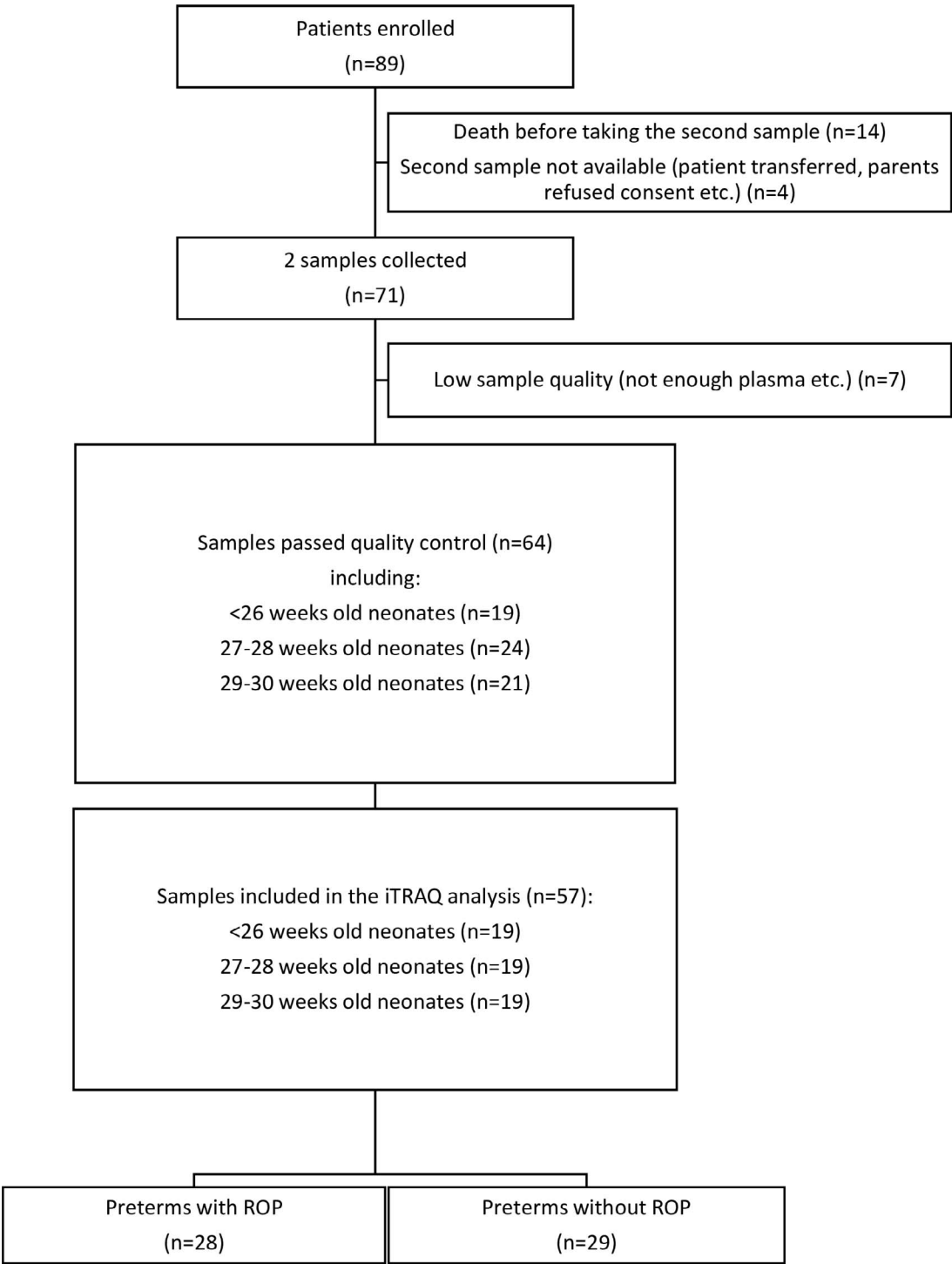


FIGURE. Flowchart showing samples included in the iTRAQ analysis.

at 36 PMA, elucidating the proteomic profile that predisposes a child to ROP.

Complement is an important component of the innate immune system, crucial for defense against microbial infections and for clearance of immune complexes and injured cells. Under normal conditions, its activation is tightly controlled and relies on pathogen-binding antibodies (classical pathway and lectin pathway), apart from low-level continuous activation caused by spontaneous C3 hydrolysis (alternative pathway).<sup>28</sup> When hyper-activated, it drives a severe inflammatory

response in numerous organs. Complement C3 is a central component of the complement cascade. Overactivation of the complement cascade has been implicated in the disease progression of glaucoma and diabetic retinopathy and is now known to be a central driver in the pathogenesis of age-related macular degeneration (AMD).<sup>29,30</sup> Complement C1 and C3 elevation in the plasma have been reported as diabetic retinopathy markers.<sup>31</sup> In a quantitative proteomic analysis using iTRAQ of murine retina with oxygen-induced retinopathy versus healthy retinas, performed by Zhou et al.,<sup>32</sup> higher levels



**TABLE 2.** Baseline Differences in Cord Blood Plasma Proteome Among Infants Who Developed ROP vs. Those Who Did Not

UniProt Protein Name	UniProt Accession No.	Ratio	<i>P</i> for Multiple Comparisons	Ratio Adjusted for GA	<i>P</i> Adjusted for GA and Multiple Comparisons
Alpha-1-antichymotrypsin	P01011	1.1862517	0.000280595	1.050	ns
Alpha-2-antiplasmin	P08697	1.1100158	0.005351421	1.047	ns
Alpha-2-macroglobulin	P01023	0.918113	8.59E−05	1.097	ns
Alpha-fetoprotein	P02771	1.0875556	0.006899515	1.251	ns
Apolipoprotein A-I	P02647	0.9224167	0.000826357	0.898	ns
Apolipoprotein A-II	P02652	0.8708346	0.043272716	0.990	ns
Apolipoprotein A-IV	P06727	0.8715872	1.84E−07	0.963	ns
Apolipoprotein D	P05090	0.9141908	0.020824291	0.977	ns
Apolipoprotein E	P02649	1.1210865	0.019551286	1.178	ns
Carboxypeptidase N subunit 2	P22792	0.9143531	0.030350218	0.829	ns
Clusterin	P10909	1.0736791	0.043818022	1.009	ns
Coagulation factor X	P00742	1.1286653	0.046620218	1.171	ns
Complement C3	P01024	1.0935421	1.99E−06	1.270	0.001
Corticosteroid-binding globulin	P08185	1.1215194	0.008725224	1.050	ns
Fibrinogen alpha chain	P02671	1.0704448	0.014554203	1.246	0.001
Gelsolin	P06396	0.943472	0.046168199	0.853	ns
Haptoglobin	P00738	1.3988051	9.71E−05	1.216	ns
Hemoglobin subunit beta	P68871	0.8777652	0.02075909	0.793	ns
Hemopexin	P02790	1.2818429	1.67E−08	1.103	ns
Hyaluronan-binding protein 2	Q14520	1.1208541	0.048320148	1.034	ns
Immunoglobulin heavy constant gamma 1	P01857	0.809804	0.000197058	0.766	ns
Immunoglobulin heavy constant gamma 2	P01859	0.847571	0.015480447	0.722	ns
Immunoglobulin heavy constant gamma 3	P01860	0.8532499	0.000465464	0.905	ns
Immunoglobulin kappa constant	P01834	0.8879205	0.025928432	0.890	ns
Immunoglobulin lambda-like polypeptide 5	B9A064	0.841204	0.000385047	0.740	ns
Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	1.1757714	0.00022227	1.093	ns
Lumican	P51884	0.9033466	0.000652683	0.959	ns
Phosphatidylcholine-sterol acyltransferase	P04180	1.2675799	0.000599877	1.155	ns
Pigment epithelium-derived factor	P36955	0.9346042	0.026092851	0.798	ns
Serotransferrin	P02787	0.8758036	4.86E−08	0.934	ns
Serum paraoxonase/arylesterase 1	P27169	0.8855303	0.034710754	0.917	ns
Vitronectin	P04004	1.1049064	0.004711251	0.978	ns

GA, gestational age; ns, not significant.

of C3 and C4 were found in murine retinopathic retinas. It is possible that complement was deposited in retinas after breaking the blood-retinal barrier, as a consequence of retinal hemorrhage.<sup>32</sup> Of note, the vitreous of diabetic patients with proliferative retinopathy also contains more complement factors than patients without retinopathy or with nonproliferative retinopathy. This observation is also explained by researchers as a breakage of a blood-retinal barrier<sup>33</sup>; however, the level of plasma complement in these patients was not checked. Rath et al.<sup>34</sup> showed an increased level of C3 (and also C4 complement component) in the vitreous and tear samples of babies with ROP in comparison with infants with congenital cataract. This study indicated a possible involvement of the alternative complement pathway in ROP. According to the authors, the intraocular complement cascade activates macrophages/microglia, which secretes proangiogenic proteins and diminishes angiogenesis inhibitor levels. That may lead to increased vessel proliferation and extracellular matrix degeneration, in turn promoting angiogenesis.<sup>34</sup> On the contrary, in a study by Langer et al.<sup>35</sup> of a murine model of ROP, mice deficient in a C3 complement component displayed increased neovascularization; moreover, an antibody-mediated blockade of other complement factors resulted in enhanced pathological retinal angiogenesis.<sup>35</sup> Our study adds further evidence to the relationship between elevated complement levels and the development of one of the ischemic retinopa-

thies, which is ROP. However, the exact connection has to be established in a targeted study. It is possible that chronic inflammatory abnormalities occurring in premature infants, caused by infections or prolonged oxygen use and excessive free radical formation, or even in the absence of well-defined triggers, may be reflected by an increased plasma C3 complement component level.

Fibrinogen is a 340-kD plasma protein synthesized by hepatocytes and is essential for blood coagulation. It is composed of two sets of three subunits, alpha, beta, and gamma. Under physiologic conditions, fibrinogen is found in plasma at low levels. However, after injury or endothelial cell dysfunction and/or vessel leakage, fibrinogen levels increase in the plasma. Fibrinogen is also a pleiotropic signaling molecule with numerous roles that can tip the balance between hemostasis and thrombosis and coagulation and fibrosis. Fibrinogen has also been shown to play a central role in inflammation.<sup>36</sup> Growing evidence indicates that perinatal inflammation and infection may increase the risk of ROP by direct proangiogenic effects and/or modifying known risk factors.<sup>37–41</sup> According to Christensen et al.,<sup>42</sup> increased fibrinogen levels may be associated with in utero infection/inflammation. On the contrary, Mitra et al.<sup>43</sup> stated in their meta-analysis that chorioamnionitis cannot be definitively considered as a risk factor for ROP. In our study, the difference in frequency of chorioamnionitis in each group, assessed both

**TABLE 3.** Differences in Plasma Proteome Among Infants Who Developed ROP vs. Those Who Did Not\*

UniProt Protein Name	UniProt Accession No.	Ratio	P for Multiple Comparisons	Ratio Adjusted for GA	P Adjusted for GA and Multiple Comparisons
Actin, alpha cardiac muscle 1	P68032	1.1906644	0.0475514	1.056	ns
Afamin	P43652	1.0799864	0.0315553	1.123	ns
Alpha-1-antichymotrypsin	P01011	0.907247	0.036339	0.917	ns
Alpha-1-antitrypsin	P01009	0.9434103	0.0286758	1.055	ns
Alpha-2-macroglobulin	P01023	1.06327	0.0044039	1.12	ns
Alpha-fetoprotein	P02771	1.170529	2.37E-05	1.17	ns
Antithrombin-III	P01008	1.1046542	0.0403643	1.085	ns
Apolipoprotein C-II	P02655	0.8994437	0.0066565	0.926	ns
Apolipoprotein D	P05090	0.9043838	0.0070745	0.922	ns
CD44 antigen	P16070	0.9067142	0.0468983	1.018	ns
Clusterin	P10909	1.1138118	0.0026749	1.146	ns
Complement C3	P01024	1.0535554	0.0092711	1.294	0.01
Corticosteroid-binding globulin	P08185	1.1463512	0.0018683	1.193	ns
Fibrinogen alpha chain	P02671	1.1117484	6.27E-05	1.304	0.001
Fibrinogen beta chain	P02675	1.0837173	0.0042849	1.196	0.05
Fibrinogen gamma chain	P02679	1.1369312	0.0001472	1.209	0.01
Hemopexin	P02790	0.9106301	0.0261357	1.02	ns
Immunoglobulin heavy constant gamma 1	P01857	0.8770847	0.0416061	1.118	ns
Immunoglobulin kappa constant	P01834	0.8573033	0.0087608	0.992	ns
Immunoglobulin lambda-like polypeptide 5	B9A064	0.8269632	0.0004377	0.978	ns
Inter-alpha-trypsin inhibitor heavy chain H2	P19823	0.9218176	0.0006118	1.031	ns
Inter-alpha-trypsin inhibitor heavy chain H3	Q06033	0.9196707	0.0127338	0.986	ns
L-selectin	P14151	0.8711616	0.0025764	0.991	ns
Leucine-rich alpha-2-glycoprotein	P02750	0.828691	7.82E-05	0.782	ns
Lumican	P51884	0.8680893	5.75E-07	0.914	ns
Osteopontin	P10451	0.882484	0.0363684	0.998	ns
Phosphatidylcholine-sterol acyltransferase	P04180	1.1502718	0.0415306	1.151	ns
Plasma protease C1 inhibitor	P05155	0.905963	0.0002958	0.992	ns
Prothrombin	P00734	1.0878711	0.0039182	1.204	ns
Zinc-alpha-2-glycoprotein	P25311	1.0855388	0.0113501	1.145	ns

GA, gestational age; ns, not significant.

\* Assessed at 36 PMA.

clinically and histologically, did not reach the statistical significance; therefore, we cannot regard intrauterine infection as the only reason for the elevated level of fibrinogen.

In a murine model of ROP, fibrinogen deposits were seen in retinas, indicating an incompetent blood-retinal barrier and a leak of this big molecule from the blood into the retina, suggesting an inflammatory response inside of the eye.<sup>44</sup> Of note, an elevated level of fibrinogen was demonstrated as a risk factor in other non-ROP ischemic retinopathies, such as diabetic retinopathy and AMD. An increased plasma fibrinogen level is an independent factor associated with the existence of diabetic retinopathy. Coagulation and fibrinolysis disturbances are significantly associated with diabetic retinopathy and exist at the early stage of microangiopathy.<sup>45–47</sup> An increased level of fibrinogen alpha chain in the vitreous was observed in patients with exudative AMD.<sup>48</sup> A higher plasma fibrinogen level was also observed in patients with AMD.<sup>49</sup> Until now, higher plasma fibrinogen levels were not directly linked with a higher

risk of developing ROP. Based on the results of our study, we believe that it is worth researching this issue in more detail.

To independently confirm iTRAQ results at a different regulatory level (RNA) with the proteome results, we also compared the expression of RNA in peripheral blood mononuclear cells (PBMCs) for the C3 complement component. It turned out that it is consistent with the results of the proteome analysis, that is it is higher in PBMCs from ROP(+) children. As the gene for the C3 complement component is expressed to some extent in the PBMCs,<sup>50</sup> it can conceivably be presumed that PBMCs may have some minor contribution to increased plasma C3 concentration. However, for fibrinogen, our microarray results do not confirm the results obtained in the proteome study. It may be connected with the fact that the main source of fibrinogen is the liver and the proportion of fibrinogen expression in leukocytes and liver is 9:>1000000.<sup>51</sup> Therefore, the expression of fibrinogen in PBMCs does not reflect its plasma concentration. But, taking into consideration

**TABLE 4.** Analysis of the Expression of Fibrinogen Alpha and C3 Complement Component Genes

Gene	Cord Blood, n = 57		36 PMA Peripheral Venous Blood, n = 57	
	Fold Change ROP(+) vs. ROP(–)	P	Fold Change ROP(+) vs. ROP(–)	P
fibrinogen alpha chain	1.062	0.38	0.969	0.54
complement component 3	1.100	0.013	1.087	0.032

the results of the studies by Hoppe and colleagues,<sup>52,53</sup> we believe that it may be possible in the future to investigate targeted therapy to reduce the production of fibrinogen in the liver, which may act in a protective way on retinal capillary beds.

### Limitations

The specific characteristics of the studied population might have influenced the results that were obtained; namely, that they were biased toward a statistically significant difference. The patient population may not be applicable to other NICUs (for example, the ROP rate), and, hence, our results may not be easily generalizable. Moreover, due to the nature of our study, there may be a potential collider bias in controlling for gestational age when low gestational age is highly associated with preterm birth, which is linked with ROP and potentially pathophysiologic factors. These issues are difficult, and sometimes unavoidable, in observational studies that are necessary when studying prematurely born children. Additionally, the validation of proteomic results by another method would strengthen the iTRAQ quantitation results. It has to be stated, however, that the sample consuming the ProteoMiner plasma enrichment allowed us only to meet the requirements for reliable iTRAQ measurements. In addition, the extensive fractionation of the labeled peptides by off-line two-dimensional chromatography and mass spectrometry provided the sensitivity of the plasma proteins analysis that would probably not be reached by the Western blot technique, which is still considered to be the standard method for targeted protein quantitation.

### CONCLUSIONS

To sum up, higher levels of complement C3 component and fibrinogen present in the cord blood that persistent to 36 PMA may indicate a chronic low-grade systemic inflammation and hypercoagulable state, which may play a role in the development of ROP.

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